



# Telomere components as potential therapeutic targets for treating microbial pathogen infections

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In a number of microbial pathogens that undergoes antigenic variation to evade the host's immune attack, genes encoding surface antigens are located at subtelomeric loci, and recent studies have revealed that telomere components play important roles in regulation of surface antigen expression in several of these pathogens, indicating that telomeres play critical roles in microbial pathogen virulence regulation. Importantly, although telomere protein components and their functions are largely conserved from protozoa to mammals, telomere protein homologs in microbial pathogens and humans have low sequence homology. Therefore, pathogen telomere components are potential drug targets for therapeutic approaches because first, most telomere proteins are essential for pathogens' survival, and second, disruption of pathogens' antigenic variation mechanism would facilitate host's immune system to clear the infection.

**Keywords:** telomere, virulence, microbial pathogen, antigenic variation, infectious diseases

## INTRODUCTION

Telomeres are nucleoprotein complexes located at the ends of linear chromosomes. In most eukaryotic cells, telomere DNA consists of simple repetitive TG-rich sequences and is maintained by telomerase, a ribonucleoprotein that contains both a catalytic protein subunit and an RNA component providing the template for *de novo* telomere DNA synthesis (Greider and Blackburn, 1987).

A number of proteins have been identified that specifically associate with the telomere DNA. In mammalian cells, the core telomere protein complex termed "Shelterin" (de Lange, 2005) contains two duplex TTAGGG repeat binding factors, TRF1 and TRF2 (Chong et al., 1995; Bilaud et al., 1997; Broccoli et al., 1997), a single-stranded telomere DNA binding protein, POT1 (Bauermann and Cech, 2001), and RAP1 (Li et al., 2000), TIN2 (Kim et al., 1999), and TPP1 (Houghtaling et al., 2004; Liu et al., 2004; Ye et al., 2004) that interact with TRFs or POT1. In addition, a trimeric CST complex containing CTC1, STN1, and TEN1 has also been identified to bind the single-stranded telomere DNA (Miyake et al., 2009; Wan et al., 2009). Fission yeast has a very similar telomere protein complex (Miyoshi et al., 2008), and many telomere protein homologs have been identified in budding yeast, too (Lewis and Wuttke, 2012). Recent studies have led to the identification of TRF (Li et al., 2005) and RAP1 (Yang et al., 2009) homologs in *Trypanosoma brucei*, a protozoan parasite belongs to the kinetoplastids group, suggesting that the telomere complex is largely conserved from protozoan to mammalian cells. Telomeres, together with their associated protein components, form a specialized structure so that the natural chromosome ends are properly protected (Stewart et al., 2011), while maintenance of a stable length of telomere DNA provides adequate docking sites for telomere binding proteins. Therefore, telomeres are essential for genome stability and sustained cell proliferation.

Although telomeres are predominantly maintained by telomerase in most eukaryotes, DNA homologous recombination can also serve as an important means for telomere maintenance (McEachern and Haber, 2006; Nabetani and Ishikawa, 2011). In addition, subtelomeric DNA recombination appears to be a major factor for genome plasticity, which may help to diversify the sequences of subtelomeric genes (Corcoran et al., 1988; Pologe and Ravetch, 1988; De Bruin et al., 1994; Louis, 1995). For several microbial pathogens whose virulence genes are located next to telomeres, this can also be an important pathogenesis mechanism to enhance their virulence (see below).

A telomere position effect (TPE) phenomenon has been observed in a number of organisms, where the expression of genes located at subtelomeres is suppressed by the nearby telomere chromatin structure (Gottschling et al., 1990; Baur et al., 2001; Koering et al., 2002; Park et al., 2002; Pedram et al., 2006). TPE is well studied in *S. cerevisiae*, where ScRap1 binds the duplex telomere DNA (Longtine et al., 1989). Both ScRap1 and yKu (a heterodimer complex that binds DNA ends in a sequence-independent manner; Riha et al., 2006) can recruit the Sir4 silencer to the telomere, and ScRap1 can also recruit Sir3 (Moretti et al., 1994; Martin et al., 1999; Moretti and Shore, 2001; Luo et al., 2002). Together, Sir3 and Sir4 recruit Sir2 (Moazed et al., 1997; Strahl-Bolsinger et al., 1997; Buchberger et al., 2008; Martino et al., 2009), which is an NAD<sup>+</sup>-dependent histone deacetylase (Tanny et al., 1999; Landry et al., 2000) and can remove the acetyl group from histone H3 at K9 and K14 residues and from histone H4K16 (Imai et al., 2000). Sir2 activity and the interaction between Sir3/4 and histone tails are necessary for establishing and propagating of the heterochromatic structure from telomere to chromosome internal regions (Hoppe et al., 2002). Similarly, TPE in human cells appears to be mediated by the heterochromatic chromatin structure, as treatment with

Trichostatin A, an inhibitor of class I and II histone deacetylases, led to decreased TPE (Koering et al., 2002).

### ANTIGENIC VARIATION AND PHENOTYPIC SWITCH IN MICROBIAL PATHOGENS

Many microbial pathogens that infect mammals have adopted antigenic variation to avoid eradication by the host immune system so that they can maintain persistent infections and enhance the chances of being transmitted to new hosts. Antigenic variation is the phenomenon that a pathogen changes its surface antigen presented to the host immune system regularly and much more frequently than spontaneous gene mutation. The term of antigenic variation usually encompasses both phase variation and true antigenic variation. In phase variation (such as phenotypic switching), the expression of an individual antigen switches between “on” or “off” states. Multiple genes from the same gene family can be expressed at the same time, and each gene’s expression state is relatively independent to that of other genes in the same family. Phenotypic switching can contribute to the virulence of the pathogen because expressing different types or various number of surface molecules may enhance or weaken adhesion of the pathogen to the host. In true antigenic variation, a certain antigen switches among different forms. The antigen is usually expressed in a mutually exclusive manner – a single gene from a multi-copy gene family is expressed at any time. In general, both antigenic variation and phenotypic switching can occur through two general types of mechanisms: genetic and epigenetic (Deitsch et al., 2009). A genetic event involves changes in DNA sequences of an antigen encoding gene or its regulatory elements so that either its expression level or its gene product is changed. An epigenetic event only affects a gene expression level but does not change its DNA sequences. However, recent studies suggest that epigenetic changes such as chromatin remodeling may also influence genetic events such as DNA recombination (Benetti et al., 2007; Bisht et al., 2008). Common mechanisms of antigenic variation have evolved in different pathogens, including bacteria, fungi, and parasites, possibly due to similar selection pressure exerted from the mammalian immune responses. However, in this chapter, we will focus on those mechanisms that are influenced or likely to be affected by the telomere structure.

### TPE PARTICIPATES IN THE REGULATION OF EPA EXPRESSION IN *C. glabrata*

*Candida glabrata* is part of the normal human mucosal flora and usually commensal, but it can cause opportunistic mucosal and bloodstream infections in immunocompromised individuals. During infection, binding of the pathogen to host cell proteins or microbial competitors would help to reduce the chance of clearance by the host. Therefore, the adherence of *C. glabrata* to host cells has been proposed to play an important role in its virulence (Kaur et al., 2005).

When cultured human epithelial cells are used, 95% of *in vitro* *C. glabrata* adherence depends on an adhesin molecule that binds the host *N*-acetyl lactosamine-containing glycoconjugates (Castano et al., 2005) and is encoded by the *EPA1* gene (Kapteyn et al., 1999), which belongs to the *EPA* gene family. So far, a total of 23 putative *EPA* genes and pseudogenes have been identified in

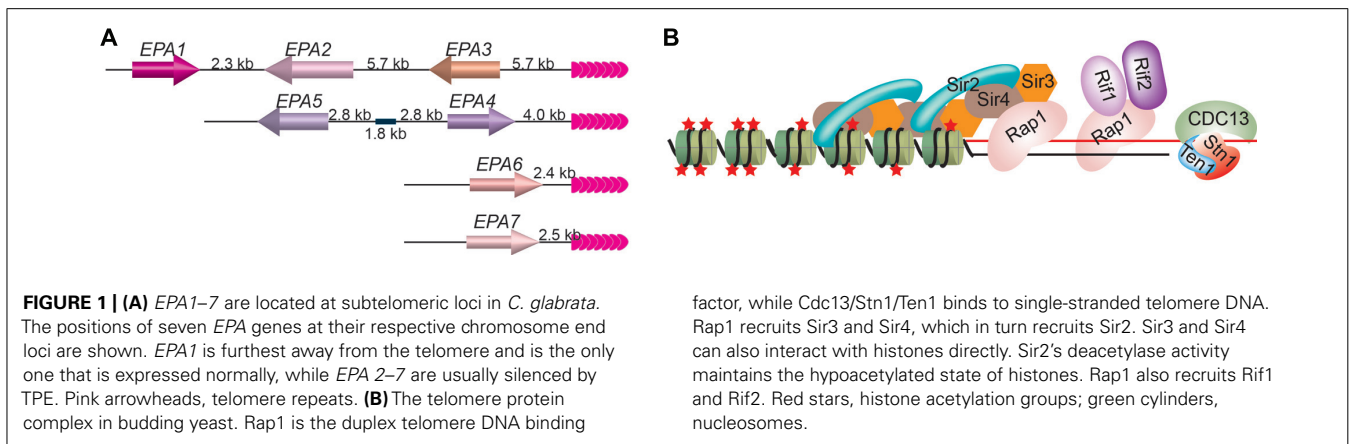
*C. glabrata* strain BG2 based on their sequence similarity (Kaur et al., 2005). Seven *EPA* genes encode full-length GPI-anchored proteins, among which *Epa1* is a lectin (Cormack et al., 1999), *Epa6* and *Epa7* are confirmed to be adhesins (Castano et al., 2005), and *Epa2* and *Epa3* are predicted to be cell wall proteins (De Las Penas et al., 2003). All seven *EPA* genes located at subtelomeric regions (Figure 1; De Las Penas et al., 2003; Castano et al., 2005; Iraqui et al., 2005).

Normally, only *EPA1* gene is active, while *EPA2–7* genes are silenced by TPE, which depends on telomere protein Rap1. Deletion of the C-terminal 28 amino acids of Rap1 led to derepression of *EPA4–7* and sometimes also *EPA2* and *EPA3* (De Las Penas et al., 2003). Silencing of subtelomeric *EPA* genes also depends on Sir proteins (De Las Penas et al., 2003; Castano et al., 2005). Deletion of *SIR3* led to hyper expression of *EPA1* and derepression of *EPA2–7*, although the derepression of *EPA2*, *EPA3*, and *EPA4/5* is mild. Deletion of *SIR4* also led to derepression of *EPA6*. In the case of deletion of *SIR3*, expression of *EPA6* and *EPA7* appears to contribute to the hyper-adherent phenotype, indicating that TPE can be directly involved in regulation of pathogen virulence (Castano et al., 2005). Interestingly, *Epa6* expression is associated with the ability of *C. glabrata* cells to form biofilm on plastic surface (Iraqui et al., 2005). Biofilm formed by microbial pathogens can increase infection probability and is of great clinical importance because microorganisms adopting this life form is more tolerant or resistant to host defense machinery and anti-microbial agents than free cells.

This TPE regulated adhesin expression is well exploited by *C. glabrata* to adapt to the host environment. *C. glabrata* is a nicotinic acid (NA or vitamin niacin) auxotroph, as it lost all the *BNA* genes involved in the NA synthesis except *BNA5* (Domergue et al., 2005). When growing in urine, where NA is limited, the activity of Sir2, an NAD<sup>+</sup>-dependent histone deacetylase, decreases correspondingly since NA is the precursor of NAD<sup>+</sup>. As a consequence, TPE level decreases, and *EPA1*, 6, and 7 genes are highly expressed (Domergue et al., 2005). This effect can be reverted by adding NA or a related compound nicotinamide (NAM). Most importantly, when using an established murine model of urinary track infection, transurethrally inoculated *C. glabrata* has an elevated colonization frequency in bladder and kidney, which is dependent on *EPA1*, 6, and 7 gene expression, and mice fed with high-NA diet are no longer susceptible to high rate of colonization of *C. glabrata* (Domergue et al., 2005). Therefore, in *C. glabrata*, TPE plays an important role in regulation of virulence gene expression.

### Sir2-MEDIATED TPE PLAYS AN ESSENTIAL ROLE IN MANOALLELIC EXPRESSION OF *var* GENES IN *P. falciparum*

*Plasmodium falciparum* is a protozoan parasite in the Apicomplexa phylum that causes the most severe form of malaria, which is a debilitating and sometimes fatal disease mostly found in tropical and subtropical regions of the world. During *P. falciparum* infection in a human host, the parasite invades first hepatocytes then erythrocytes. One major reason why it is very difficult to eliminate these parasites once an infection is established is that *P. falciparum* undergoes antigenic variation at the erythrocyte stage (Dzikowski and Deitsch, 2009). At this stage, *P. falciparum* cells produce



erythrocyte membrane protein 1 (PfEMP1), which is encoded by *var* genes and is transported to the infected erythrocyte membrane (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). Expression of PfEMP1 on the infected cell surface allows the infected erythrocyte to adhere to the endothelium of the post-capillary venules and avoid circulation through the spleen, where the infected cells will be destroyed (Baruch, 1999). Therefore, expression of PfEMP1 on host cell surface is critical for prolonged parasite infection. However, PfEMP1 is also susceptible to host antibody recognition and subsequent immune attack. As an important virulence mechanism, *P. falciparum* regularly switches the expressed PfEMP1, therefore effectively evading the host immune attack (Roberts et al., 1992).

There are ~60 *var* genes in the *P. falciparum* genome (Gardner et al., 2002). However, only one *var* gene is expressed at any moment (Roberts et al., 1992). Based on its upstream regulatory elements, *var* genes can be classified into three groups (Figure 2; Kraemer and Smith, 2003; Lavstsen et al., 2003). Those with UpsA and transcribed toward the telomere and those with UpsB and transcribed away from the telomere are located at subtelomeric loci (Figure 2A), while the ones with UpsC are located at chromosome internal loci (Figure 2B; Voss et al., 2000; Gardner et al., 2002; Kraemer and Smith, 2003; Lavstsen et al., 2003). Monoallelic expression of *var* gene is regulated at multiple levels, and telomeres appear to play an important role (Dzikowski and Deitsch, 2009).

Telomere position effect in *P. falciparum* spreads ~55 kb along the chromosome from telomeres and was first observed by targeting a reporter gene to the rep20 repeats located at the subtelomeric regions (Figure 2A; Duraisingh et al., 2005). Rep20 is the most telomere-distal telomere associated repetitive element (TARE) and is usually adjacent to the subtelomeric *var* gene promoter. TPE in *P. falciparum* depends on Sir2 (Duraisingh et al., 2005; Freitas-Junior et al., 2005; Tonkin et al., 2009), which is both a histone deacetylase and an ADP-ribosyltransferase (Merrick and Duraisingh, 2007; Chakrabarty et al., 2008) and is localized at the telomeres, where histones H4 acetylation is absent (Freitas-Junior et al., 2005). By examining subnuclear localization of a number of genetic markers along chromosome 2 by FISH, it is also inferred that chromatin structure is more condensed for telomere-proximal regions than telomere-distal ones (Freitas-Junior et al., 2005). The

factor, while Cdc13/Stn1/Ten1 binds to single-stranded telomere DNA. Rap1 recruits Sir3 and Sir4, which in turn recruits Sir2. Sir3 and Sir4 can also interact with histones directly. Sir2's deacetylase activity maintains the hypoacetylated state of histones. Rap1 also recruits Rif1 and Rif2. Red stars, histone acetylation groups; green cylinders, nucleosomes.

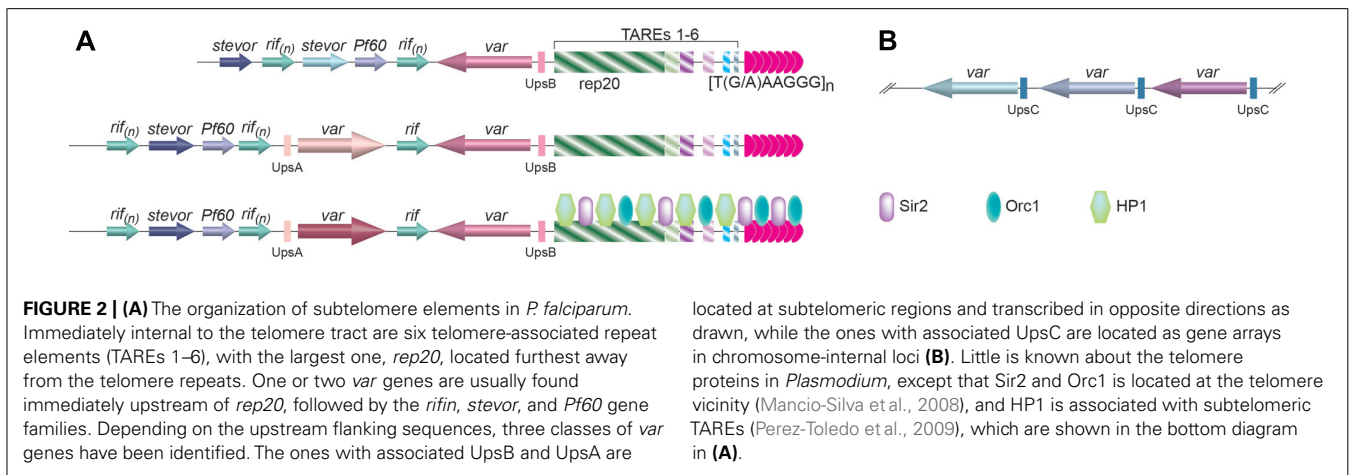
direct evidence of involving TPE in *var* gene regulation came from the observation that deletion of PfSir2 led to a significant increase in transcription of a subset of *var* genes, particularly the *var* genes with UpsA and at the subtelomere regions (Duraisingh et al., 2005).

### RAP1-MEDIATED SILENCING IS ESSENTIAL FOR MONOALLELIC EXPRESSION OF VSG IN *T. brucei*

The kinetoplastids are a group of flagellated protozoa. Three members of kinetoplastids are of great clinical importance because they cause human diseases: *Trypanosoma brucei* causes human African trypanosomiasis or sleeping sickness, *Trypanosoma cruzi* causes South America trypanosomiasis or Chagas disease, and several *Leishmania* species cause leishmaniasis. Of these three trypanosomatids (the kinetoplastid organisms that only have a single flagellum), only *T. brucei* undergoes antigenic variation, which is an important mechanism of its pathogenesis and one of its most interesting biological aspects (Barry and McCulloch, 2001).

*Trypanosoma brucei* is transmitted between mammalian hosts by an insect vector, tsetse (*Glossina* spp.). While inside the mid-gut of a tsetse fly, *T. brucei* cells are in the non-virulent proliferative stage, procyclic form (PF), and several procyclic acidic repetitive proteins (PARPs, or procyclins) are expressed on its surface. After *T. brucei* cells migrate into the salivary gland of the tsetse fly, they differentiate into the metacyclic form, stop proliferating, and acquire virulence. When a tsetse fly takes a blood meal, *T. brucei* cells can be injected into a mammalian host. They stay in the bloodstream or extracellular spaces in the host and quickly differentiate into bloodstream form (BF). The slender BF is proliferative, while the stumpy form is quiescent and non-proliferative. The metacyclic form and BF cells express variant surface glycoproteins (VSGs) as their major surface glycoprotein (Mehlert et al., 1998). When a tsetse fly bites the infected mammalian host, stumpy bloodstream form *T. brucei* cells taken into the midgut of a tsetse can quickly differentiate into the PF, ending the life cycle (Matthews, 2005).

*Trypanosoma brucei* is exposed to the host's immune system and is vulnerable to both the innate (inflammations, complements, etc.) and adaptive immune responses (antibody, killer T cells, etc.). However, *T. brucei* has evolved a sophisticated antigenic variation mechanism and regularly switches its surface



VSG coat, thus effectively evading the host's immune response (Barry and McCulloch, 2001).

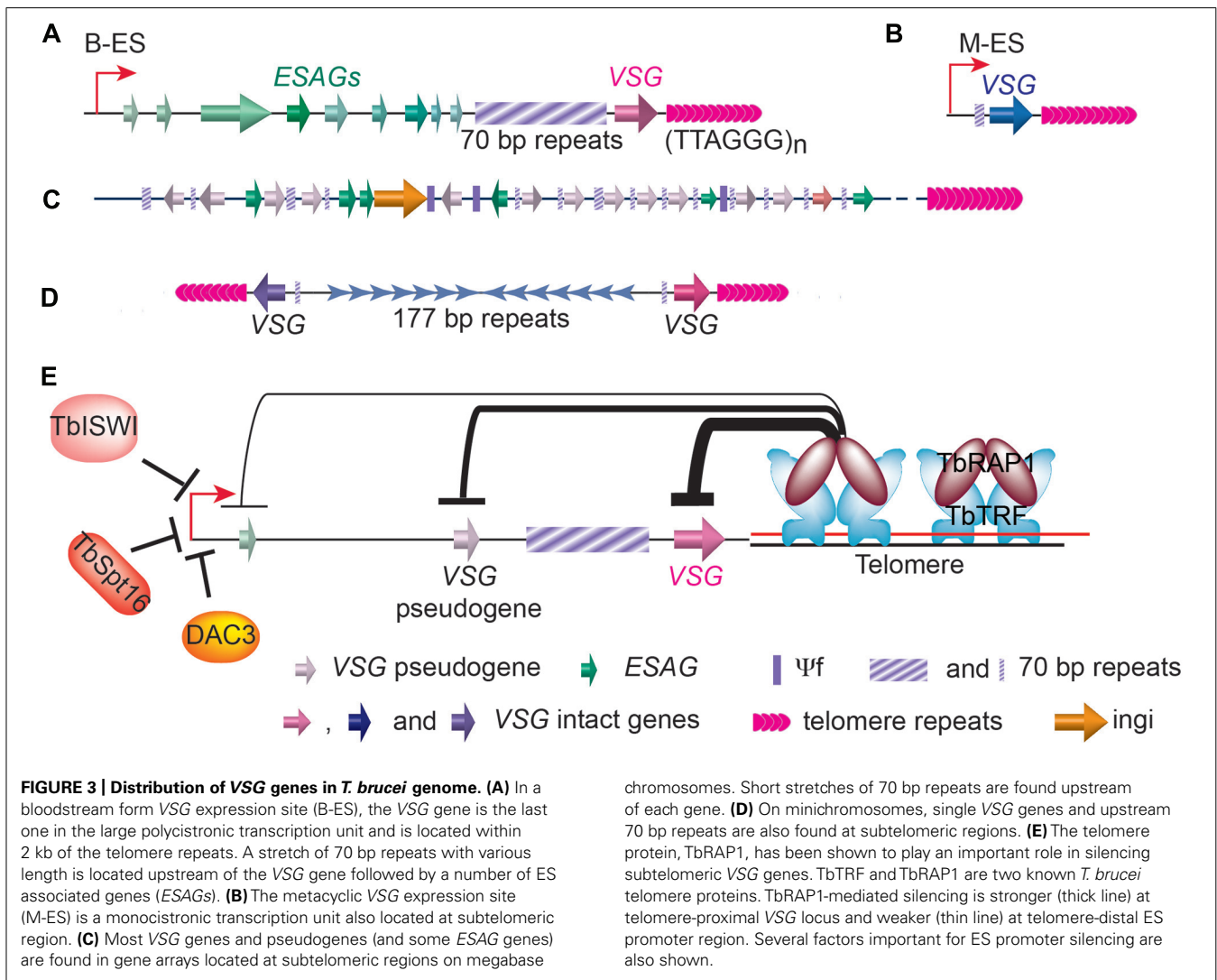
Antigenic variation in *T. brucei* has two essential aspects: switch to express a different VSG gene (VSG switching) and monoallelic expression of VSG. Although there are >1,500 VSG genes and pseudogenes in the *T. brucei* genome (Berriman et al., 2005), only one type of VSG is expressed at any time. After a new VSG gene is turned on, it is essential to turn off the previously active VSG so that the old surface antigen is no longer presented to the host immune system. In addition, expressing only one VSG gene at a time would allow the VSG gene pool to be used for a maximum period of time, enabling a persistent infection. Therefore, both VSG switching and monoallelic expression of VSG are critical for antigenic variation and have been the focus of intensive research for several decades.

There are 11 pairs of megabase chromosomes (0.9–5.7 Mb), several intermediate chromosomes (300–900 kb), and ~100 copies of minichromosomes (50–100 kb) in *T. brucei* genome (Melville et al., 2000; Alsford et al., 2001; Berriman et al., 2005). The majority of VSG genes are found in long tandem arrays of repeated genes at subtelomeric locations on megabase chromosomes (Figure 3C). Approximately 200 copies of VSG genes are found immediately upstream of telomeres of the minichromosomes, which carry besides the VSG genes, only repetitive sequences, including 177 bp repeats in the chromosome internal region and telomere repeats at the chromosome ends (Figure 3D; Alsford et al., 2001). The VSG genes in subtelomeric gene arrays and on minichromosome are often referred to as basic VSG copies because they are transcriptional silent. The rest of VSGs are found in VSG expression sites. In BF *T. brucei* cells, VSGs are expressed exclusively from bloodstream form VSG expression sites (B-ESs) which are RNA polymerase I (RNAP I)-transcribed, polycistronic transcription units located at subtelomere loci (Figure 3A; de Lange and Borst, 1982; Gunzl et al., 2003). VSG is the last gene in any B-ES and is usually within 1.5 kb from the telomere repeats, while the promoter is often 40–60 kb upstream of the VSG (Hertz-Fowler et al., 2008). In contrast, at the metacyclic stage, VSGs are expressed from metacyclic VSG expression sites (M-ESs), which are monocistronic transcription units located at the subtelomeric regions (Figure 3B), with the promoter located only ~5 kb

from the telomere (Lenardo et al., 1984; Cornelissen et al., 1985). Although the M-ESs have much simpler organizations than the B-ESs, much less is understood about metacyclic than bloodstream VSG expression regulation. *T. brucei* has multiple B-ESs (e.g., Lister 427 has 15 different B-ESs), usually carrying different VSGs, but all B-ESs have very similar genomic organization with ~90% sequence identity (Hertz-Fowler et al., 2008). Earlier studies focused on B-ES promoters also showed that they are almost always identical (Zomerdijk et al., 1990, 1991; Pham et al., 1996). Therefore, how *T. brucei* manages to fully express only one B-ES and VSG had been a great puzzle for more than a couple of decades.

A number of studies in the last decade have shown that VSG expression is regulated at multiple levels. First, transcription elongation from B-ES promoter appears to be regulated. Silent B-ES promoters are actually mildly active (Vanhamme et al., 2000), but transcription elongation is quickly attenuated after a few kilobases, effectively stopping transcription long before the VSG genes. Second, chromatin structure of the active B-ES is very different from the silent B-ESs. The active B-ES has very few nucleosomes while silent B-ESs are packed with nucleosomes (Figueiredo and Cross, 2010; Stanne and Rudenko, 2010). Chromatin remodeling also plays an important role in regulating the B-ES expression: Deletion of the histone H3K79 methyltransferase TbDot1b led to a 10-fold increase in transcription throughout the silent B-ESs (Janzen et al., 2006). Additional chromatin remodelers have been shown to affect B-ES promoter but not downstream VSG expression (Figure 3E): Depletion of a Swi/Snf homolog, TbISWI, led to an elevated transcription from the silent B-ES promoters (Hughes et al., 2007; Stanne et al., 2011); DAC3, a histone deacetylase homologs, is required for B-ES promoter silencing (Wang et al., 2010); and depletion of TbSpt16, a subunit of the FACT chromatin remodeling complex, also led to an ~20-fold increase in silent B-ES promoter transcription (Denninger et al., 2010). Third, ever since the discovery that VSGs are exclusively expressed from subtelomeric regions (de Lange and Borst, 1982), it has been proposed that telomeres may play an important role in VSG expression regulation (Dreesen et al., 2007). This hypothesis was supported by the fact that TPE has been observed in *T. brucei* (Horn and Cross, 1997a; Glover and Horn, 2006).





Although the earlier studies provided promising evidence for TPE, direct evidence linking TPE and VSG silencing was lacking for a long time. In addition, although the *T. brucei* Sir2 homolog plays an essential role in TPE at reporter marked telomeres without native B-ESs, its deletion does not affect VSG silencing at all (Alsford et al., 2007). Furthermore, Glover et al. (2007) was able to target an I-Sce I digestion site together with a *neo* reporter gene downstream of the VSG gene and immediately upstream of the telomere in a telomerase null background. Induction of ectopic I-Sce I expression led to immediate cleavage and loss of the marked telomere. Within 9 h, degradation of the reporter gene and the subtelomeric VSG gene was also observed. Although a mild derepression of the reporter gene was observed shortly before it was degraded, the VSG gene was not derepressed at all (Glover et al., 2007). These observations raised a great deal of doubts whether telomeres are indeed necessary for proper VSG silencing.

It was difficult to examine the roles of the telomere in antigenic variation directly without identifying any telomere specific proteins. Earlier attempts to identify telomere DNA binding factors in *T. brucei* using biochemical approaches led to the identification of

a couple of telomere DNA binding activities without identification of the responsible proteins (Eid and Sollner-Webb, 1995, 1997).

The first *T. brucei* Shelterin homolog, TbTRF, was identified using an *in silico* approach (Li et al., 2005), and a yeast 2-hybrid screen using TbTRF as bait led to the identification of *T. brucei* RAP1 (Yang et al., 2009), another integral component of the *T. brucei* telomere complex (Figure 3E). When TbRAP1 was depleted by RNAi (Shi et al., 2000), a derepression of silent B-ES-linked VSGs can be detected (Yang et al., 2009). Using quantitative RT-PCR analysis, it was shown that all B-ES-linked silent VSGs had an elevated expression level upon depletion of TbRAP1, although the level of derepression varies among different VSGs, ranging from 8- to 56-fold. Subsequently, it was confirmed by IF that multiple VSGs are expressed simultaneously in individual cells on cell surface (Yang et al., 2009). Importantly, the TbRAP1-mediated silencing is position dependent. First, only subtelomeric B-ES-linked VSGs were affected. Genes located in chromosome internal regions including RNAP I transcribed rDNA and RNAP II transcribed telomerase protein gene, a ribosomal protein gene, and a glycolytic protein gene were not affected. Second, within an

individual B-ES, the telomere-adjacent VSG gene is derepressed at the highest level, a VSG pseudogene located 7–20 kb away from the telomere is derepressed at an intermediate level, and a reporter gene targeted immediately downstream of the B-ES promoter located 40–60 kb away from the telomere is derepressed at the lowest level (Figure 3E). It is therefore convinced that the TbRAP1-mediated silencing originates from the telomere, demonstrating for the first time that the telomere structure indeed plays an essential role in VSG expression regulation (Yang et al., 2009).

However, the involvement of telomere in VSG expression regulation does not necessarily exclude other mechanisms mentioned above. In fact, TbRAP1-mediated silencing appears to block the elongation of the basal level transcription from the silent B-ES promoters, because in TbRAP1 deficient cells, derepressed VSGs are expressed at a level that is still ~100 fold lower than when the same VSG is in a fully active B-ES (Yang et al., 2009). Therefore, the observed quick attenuation of transcription elongation along silent B-ESs may well be the combined effect of a basal level transcription initiated from silent B-ES promoters and a TbRAP1-mediated TPE. The fact that derepressed VSGs are not expressed at its fullest potential also suggests that B-ES promoters are regulated by additional factors other than TPE. This is consistent with the observations that a number of chromatin remodeling factors are involved in B-ES promoter regulation as mentioned above.

Recent studies have made great contributions to our understanding of how VSG expression is silenced. However, how is allelic-exclusive expression of VSG achieved is not fully understood. It has been proposed that sufficient amount of RNAP I machinery, which is responsible for high level VSG transcription, may be accessible to only one B-ES, which would effectively ensure its monoallelic expression (Horn and McCulloch, 2010). In an IF analysis, Navarro and Gull (2001) found that in BF *T. brucei* cells, transcriptionally active RNAP I forms a small nuclear focus in addition to the large focus inside the nucleolus, where it transcribes rRNA. In addition, only the active B-ES but not the silent ones is co-localized with RNAP I in this ES body (ESB), which only exists in BF but not PF cells (Navarro and Gull, 2001). It is therefore hypothesized that ESB, enriched with RNAP I, can only accommodate one B-ES, which would effectively limit the number of active B-ES to one. In support of this view, when two different B-ESs were tagged with selective markers immediately downstream of their respective promoters and forced to be active simultaneously, the two B-ESs appear to switch back and forth rapidly and locate next to each other in the nucleus, presumably competing for available RNAP I at ESB (Chaves et al., 1999).

#### TELOMERE LENGTH AFFECTS VSG SWITCHING FREQUENCY AND MECHANISM IN *T. brucei*

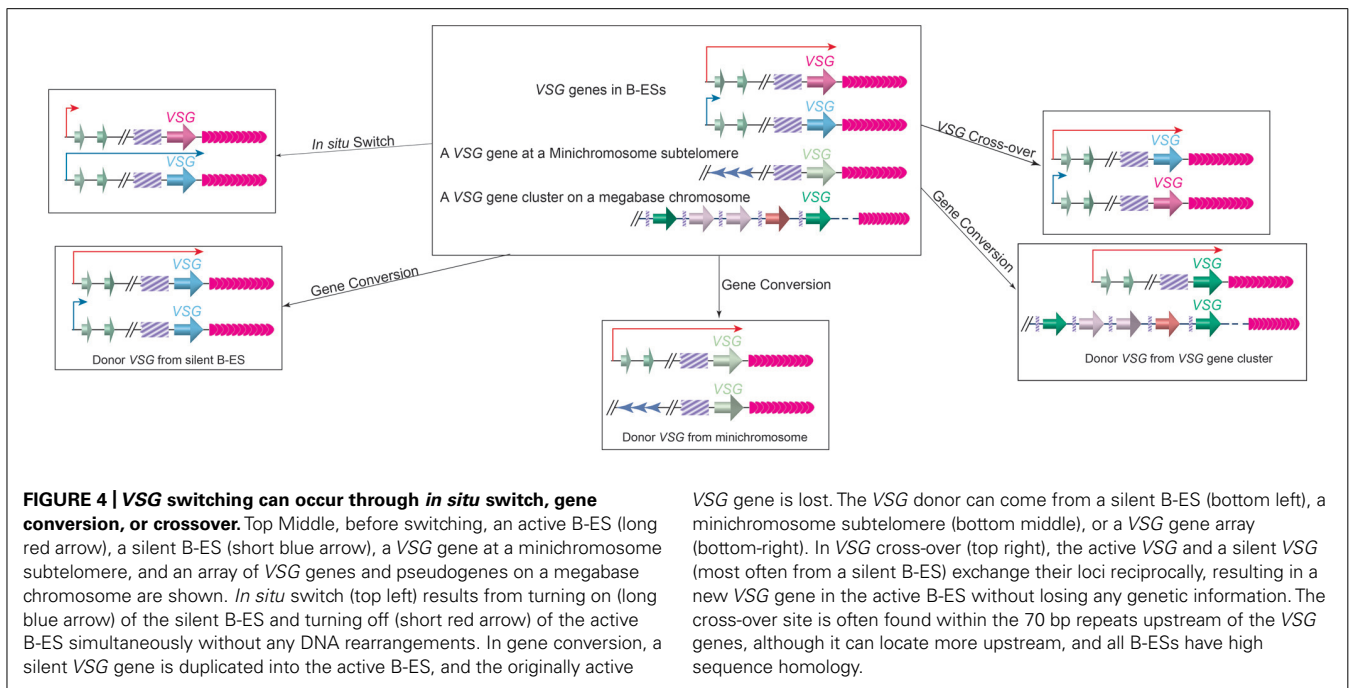
VSG switching can occur through several different pathways (Figure 4; Barry and McCulloch, 2001). In the so-called *in situ* switch, a silent B-ES promoter is turned fully active while the originally active B-ES promoter is turned off without any DNA rearrangements. There are 15 B-ESs carrying distinctive VSGs in the *T. brucei* Lister 427 cells, providing a small number of possible *in situ* switch opportunities (Hertz-Fowler et al., 2008). However, *in situ* VSG switching is usually a rare event, and VSG switching involving DNA recombination events are much more prevalent

(Robinson et al., 1999). The large VSG gene pool, therefore, provides essentially endless possibilities for VSG switching.

In gene conversion events, a silent VSG is copied into the active B-ES while the originally active VSG is lost. In this event, the donor can be any functional VSG gene in the genome. There is almost always a stretch of 70 bp repeats upstream of a VSG gene, in which homologous recombination can initiate as DNA double-strand breaks (Boothroyd et al., 2009). In rare occasions, several VSG donors have been identified in a single VSG switching event, where each donor contributes only a fragment of the gene, generating a new mosaic VSG gene product (Marcello and Barry, 2007). Such mechanism has been proposed to be useful in late stage of persistent infection. More often, a silent B-ES is used as a donor possibly because long stretch of 70 bp repeats (2 to >14 kb) and telomere repeats (3–20 kb) flank the VSG gene in any B-ES, and efficient homologous recombination can initiate from these sites. In fact, all B-ESs have very similar genome organization and are ~90% identical in sequences, so gene conversion event can initiate at places upstream of 70 bp repeats and often a whole silent B-ES can be copied to replace the active B-ES (Pays et al., 1983b; Hertz-Fowler et al., 2008). Therefore, the terms of VSG gene conversion and ES gene conversion are used to differentiate different types of gene conversion events (Kim and Cross, 2010). In addition to gene conversion, reciprocal crossover event can occur in a VSG switching (Rudenko et al., 1996). In this case, the crossover usually occurs at the 70 bp repeats, and the silent and active VSGs (often together with their respective downstream telomeres) simply trade places without deletion of large fragments of genetic information. It is worth to note that in a crossover switching, the originally silent VSG often comes from a silent B-ES, but it can also be from a minichromosome subtelomere. Finally, more complicated switching events involving loss of the active B-ES or VSG associated with an *in situ* switch have also been observed (Kim and Cross, 2011).

It has been shown that homologous DNA recombination is important for VSG switching in *T. brucei* (McCulloch and Barry, 1999). In homologous recombination, searching for DNA sequence homology and subsequent strand-invasion is a key step, at which RAD51 polymerizes around ssDNA to assemble a nucleoprotein helical filament and, with the help of ATP, extends the DNA structure and carries out the strand exchange process (Holloman, 2011). When ssDNA is coated with RPA (a single-strand-specific DNA binding protein), it will not be accessible by RAD51 without the help of a mediator, such as BRCA2 (Holloman, 2011). In *T. brucei*, six RAD51 related proteins have been identified: RAD51, DMC1, RAD51-3, RAD51-4, RAD51-5, and Rad51-6 (Proudfoot and McCulloch, 2005). Among these, deletion of TbRAD51 and TbRAD51-3 led to a decrease in VSG switching rate while deletion of TbRAD51-5 did not have any effect, and deletion of TbBRCA2 also led to a similar decreased VSG switching rate (Hartley and McCulloch, 2008). In addition, TbTOPO3 $\alpha$  and TbRMI1, whose homologs in mammalian cells form a so-called RTR complex with The RecQ helicase BLM and suppress aberrant and inappropriate homologous recombination, were recently shown to be involved in regulation of VSG switching (Kim and Cross, 2010, 2011).

Apparently, homologous recombination is a major pathway for VSG switching. However, exactly how VSG switching is regulated



VSG gene is lost. The VSG donor can come from a silent B-ES (bottom left), a minichromosome subtelomere (bottom middle), or a VSG gene array (bottom-right). In VSG cross-over (top right), the active VSG and a silent VSG (most often from a silent B-ES) exchange their loci reciprocally, resulting in a new VSG gene in the active B-ES without losing any genetic information. The cross-over site is often found within the 70 bp repeats upstream of the VSG genes, although it can locate more upstream, and all B-ESs have high sequence homology.

is less clear. Several recent studies now indicate that the telomere structure can influence VSG switching greatly.

It has been shown that the active VSG-marked telomere is less stable than the silent telomeres (Bernards et al., 1983; Pays et al., 1983a; van der Ploeg et al., 1984; Myler et al., 1988; Horn and Cross, 1997b). Rapidly shortened active telomere arises frequently, which is quite similar to the TRD observed in yeast cells carrying abnormally long telomeres (Li and Lustig, 1996). Presumably the active transcription of the telomere is a major cause for the brittle active telomere (Rudenko and Van der Ploeg, 1989). With the presence of telomerase, shortened telomeres are elongated quickly (Horn et al., 2000). With frequent truncation and elongation, telomere length at the active chromosome end is often much more heterogeneous than those at silent telomeres (Bernards et al., 1983). However, in the absence of telomerase, the truncated active telomere remains short, allowing the isolation of clones bearing extremely short active telomere in a relatively short culturing period (Dreesen and Cross, 2006). Interestingly, when such telomerase negative clones were obtained that carry extremely short active telomere, these clones tend to switch to express a new VSG (Dreesen and Cross, 2006). This observation led to the hypothesis that shorter telomeres may cause higher VSG switching rate (Dreesen et al., 2007). It is speculated that all active telomeres are prone to large telomere fragment deletions due to its active transcription state, but shorter telomeres are more likely to have a deletion landed in the subtelomeric region and to cause damage in the active VSG gene, which will force the parasite to go through VSG switching. Introducing a break at the I-SceI site targeted immediately upstream of the active VSG gene led to a 250-fold increase in VSG switching frequency, confirming part of this theory that damage to the active VSG gene will force the parasite to switch (Boothroyd et al., 2009).

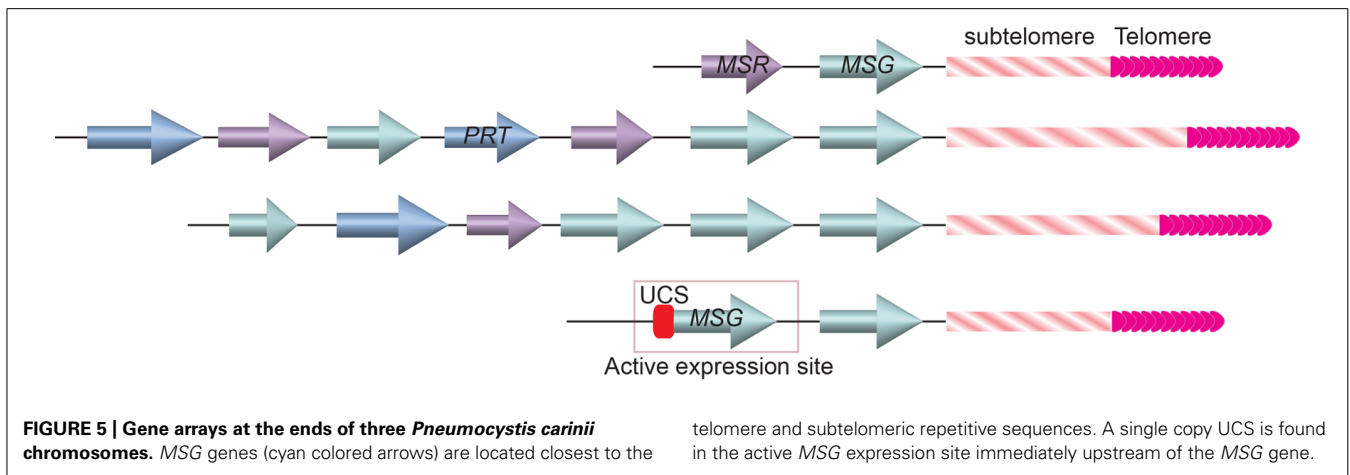
Importantly, a recent study showed that cells carrying short active telomeres (~1.5 kb) has an ~6.3-fold higher VSG switching

frequency than cells carrying long telomeres (>10 kb; Hovel-Miner et al., 2012). In addition, cells with short active telomere also have more gene conversion and much fewer telomere crossover events as VSG switching mechanism (Hovel-Miner et al., 2012). Therefore, telomere length indeed affects subtelomeric VSG switching. At least two Shelterin homologs have been identified in *T. brucei* (Li et al., 2005; Yang et al., 2009), which enabled further investigation of the telomere structure in VSG switching regulation. It is speculated that disruption of the heterochromatic telomere structure, especially in the case of depletion of TbRAP1 (Yang et al., 2009), may also lead to higher VSG switching rate, similar to what was observed in *S. pombe* (Bisht et al., 2008).

#### DOES TELOMERE AFFECT SWITCHING OF SUBTELOMERE-LOCATED SURFACE ANTIGEN IN *P. carinii* AND *B. burgdorferi*?

*Pneumocystis carinii* is a fungus that solely dwells in the lung tissue of mammals. Normally, *P. carinii* infection does not cause any symptom, but in immunocompromised individuals it can cause pneumonia. The complete life cycle of *P. carinii* is still not very well defined, mainly because of the lack of a continuous cultivation system. However, it is obvious that *P. carinii* can survive in the lower respiratory tract where strong and effective defense systems normally work to eliminate invaders, and the reason for persistent and effective *P. carinii* infection is that it undergoes antigenic variation at a high frequency (Cushion and Stringer, 2010).

The Major Surface Glycoprotein (MSG) is one of the major surface molecules of *P. carinii* that is involved in antigenic variation (Stringer, 2005). MSG is encoded by the MSG gene family. So far 73 MSG genes have been identified, all are located at the subtelomeric loci (Figure 5; Keely and Stringer, 2009). There are 17 chromosomes in *P. carinii* (Hong et al., 1990), indicating that



on average at least two *MSG* genes are at each telomere, which is often the case in cloned terminal fragments from various chromosomes (Wada and Nakamura, 1996; Keely et al., 2005). Similar to the situation in *T. brucei*, only one *MSG* gene is transcribed at any time. Transcribed *MSG* messengers always contained an upstream conserved sequence (UCS; Wada et al., 1995; Edman et al., 1996; Wada and Nakamura, 1996; Sunkin and Stringer, 1997), which has only one copy in the *P. carinii* genome (Wada et al., 1995; Edman et al., 1996), suggesting that *MSG* is transcribed from a specific expression site marked with the unique UCS element. In addition, translation initiation codon on an *MSG* mRNA is located in the sequence transcribed from the UCS (Wada et al., 1995; Edman et al., 1996). Therefore, transcribing *MSG* from UCS-containing expression site is essential for proper *MSG* translation. Furthermore, the UCS encoded peptide contains a signal sequence that targets the pre-*MSG* protein into the endoplasmic reticulum, where it can be cleaved and glycosylated, then deposited on the cell surface (Sunkin et al., 1998). Hence the UCS peptide is also essential for *MSG* function, although it is not present on *MSG* found on the cell surface because it is likely removed in the endoplasmic reticulum.

If *P. carinii* contains only one UCS-containing *MSG* expression site, how does it achieve antigenic variation? Computational analysis of *MSG* gene sequences suggested that these genes commonly undergo recombination (Wada and Nakamura, 1996; Keely et al., 2005; Keely and Stringer, 2009), which is not unlike the *VSG* switching in *T. brucei*. Similar to *VSG*, *MSG* is also the last transcribed gene on the chromosome (Wada and Nakamura, 1996; Keely et al., 2005). The proximity of *MSG* genes to telomeres suggests that the *MSG* switching events might also be regulated by the telomere structure, although this has not been investigated at all.

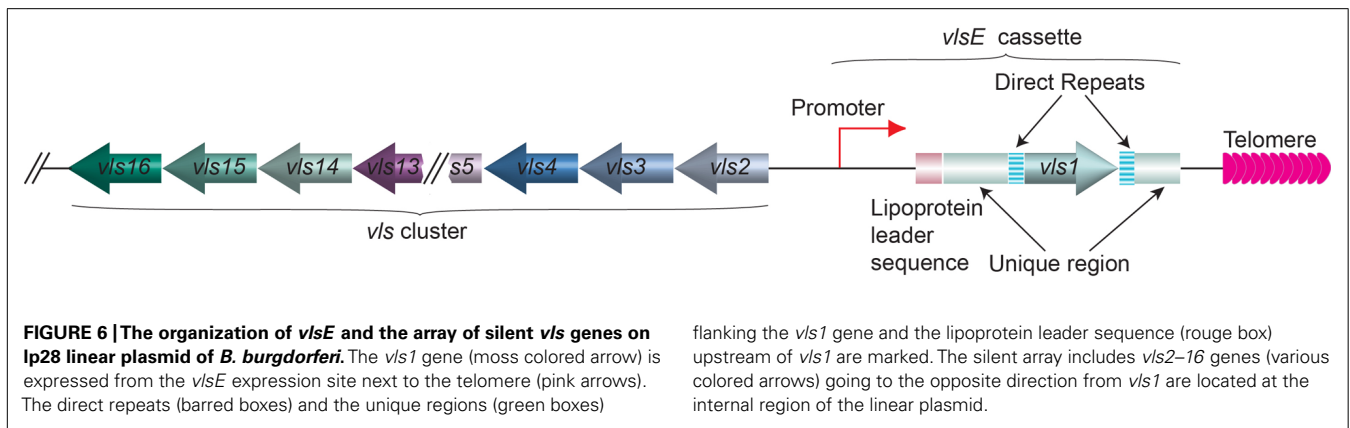
In a different microbial pathogen *Borrelia burgdorferi*, the spirochete that causes the Lyme disease, the gene encoding variant surface antigen is found at a subtelomere region on a linear plasmid (Zhang et al., 1997). *B. burgdorferi* also undergoes antigenic variation, and the lipoprotein *VlsE* is the variant surface protein (Schwan et al., 1991; Zhang et al., 1997; Zhang and Norris, 1998b; Steere et al., 2004; Norris, 2006). *VlsE* is encoded by the *vls* gene family located on the linear plasmid lp28-1 (Figure 6).

Immediately next to the telomere is the active *vlsE* expression site. More upstream is the silent *vls* gene cluster (Zhang et al., 1997). Bacteria lost the lp28-1 exhibit an intermediate infectivity phenotype where it is hard to establish a persistent infection in the mouse model (Bankhead and Chaconas, 2007). Deletion of *vlsE* and silent *vls* cassettes also led to reduced persistent infection, indicating that antigenic variation through *vls* switching is an important virulence mechanism in *B. burgdorferi* (Zhang et al., 1997; Purser and Norris, 2000; Labandeira-Rey and Skare, 2001; Bankhead and Chaconas, 2007). The *vlsE* and the silent *vls* genes are highly homologous at the sequence level, and most of the sequence differences within the cassette regions are concentrated in six variable regions, VR1–VR6 (Zhang and Norris, 1998a). Segmental gene conversion between the silent cassettes and the *vlsE* cassette region occurs as early as 4 days after infection in mice, and appears to continue throughout the course of infection (Zhang and Norris, 1998b). Because these recombination events appear to involve random segments of any silent cassette and occur continuously during infection, an almost unlimited number of *VlsE* amino acid sequence permutations are theoretically possible (Zhang and Norris, 1998a). Apparently, *vls* switching is not so unlike the *VSG* switching in *T. brucei* or *MSG* switching in *P. carinii*. However, nothing is known about the telomere structure at the ends of lp28 or any protein(s) associated with it. Therefore, it is unclear whether the nearby telomere structure might exhibit any influence to *vls* switching.

## TELOMERE COMPONENTS AS POTENTIAL TARGETS OF ANTI-PATHOGEN AGENTS

In this review, I have provided a detailed treatise of the telomere region, the adjoining genes and sites, and the regulatory elements and proteins in several microbial pathogens that undergo antigenic variation. As discussed, telomere forms a specialized heterochromatic structure that can influence the expression of genes located nearby. It appears that several microbial pathogens have conveniently taken advantage of this TPE to regulate expression of surface antigen-encoding gene families at subtelomeric regions. Further studies of the telomere structure and telomere-specific proteins in these microbial pathogens should provide more insight about the allelic exclusion expression of surface antigen genes.





In addition, the subtelomeric region in many eukaryotic cells appears to be a DNA recombination hot spot, presumably contributing to gene diversity. This could be one of the reasons why many gene families encoding virulence factors are located at subtelomeric loci in microbial pathogens. One cannot help to speculate that the intrinsic plastic nature of the subtelomeres might facilitate antigenic variation. On the other hand, unchecked homologous recombination could cause hazardous genome instability, and the telomere structure with telomere-specific proteins is hypothesized to suppress subtelomeric recombination to maintain a relatively stable genome organization.

The essential functions of telomeres in maintaining genome integrity are conserved for all eukaryotic cells, and homologs of many telomere-specific proteins have been identified from protozoa to mammals. However, telomere homologs from mammals and those from the above-mentioned microbial pathogens have very low sequence homology. As the telomere components play important functions in regulation of virulence in several microbial pathogens, they are attractive drug targets for treatment of diseases caused by these pathogens. For example, TbRAP1 and hRAP1 has very limited sequence homology. It should be feasible to identify or develop agents that specifically target TbRAP1 but not hRAP1. These agents are expected to act as a double-edged sword. First, TbRAP1 is essential for trypanosome cell growth and dysfunctional TbRAP1 leads to cell growth arrest. Second, TbRAP1 is essential for complete subtelomeric VSG silencing, and lack of TbRAP1 leads to expression of multiple VSG proteins on trypanosome cell surface, which will facilitate the host immune system to eliminate the parasite efficiently.

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